Fabrication of whey protein/pectin double layer microcapsules for improving survival of *Lacticaseibacillus rhamnosus* ZFM231

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ABSTRACT

To improve the viability of *Lacticaseibacillus rhamnosus* ZFM231 strain in the gastrointestinal tract and exhibit better probiotic effect, an internal emulsification/gelation technique was employed to encapsulate this strain using whey protein and pectin as wall materials to fabricate the double layer microcapsules. Four key factors affecting the encapsulation process were optimized using single factor analysis and response surface methodology. Encapsulation efficiency of *L. rhamnosus* ZFM231 reached 89.46±0.82%, the microcapsules possessed a particle size of 172±1.80 μm and ζ-potential of -18.36 mV. The characters of the microcapsules were assessed using optical microscope, SEM, FT-IR and XRD analysis. It was found that after exposure to simulated gastric fluid, the bacterial count (log (CFU g^{-1})) of the microcapsules only lost 1.96 units, the bacteria were released readily in simulated intestinal fluid, reaching 86.56% after 90 min. After stored at 4 °C for 28 days and 25 °C for 14 days, bacterial count of the dry microcapsules decreased from 10.59 to 9.02 and 10.49 to 8.70 log (CFU g^{-1}), respectively. The double layered microcapsules could significantly increase the storage and thermal abilities of bacteria. Such *L. rhamnosus* ZFM231 microcapsules could find applications as ingredient of the functional foods and the dairy products.

Keywords: Microcapsule; *Lacticaseibacillus rhamnosus*; Gastrointestinal conditions

1. Introduction

Lacticaseibacillus rhamnosus is a widely concerned probiotic in the lactic acid bacterial family, as it has shown the capacity to inhibit *Helicobacter pylori* infection [1], prevent intestinal damage, and improve immunity [2]. In order to achieve these probiotic effects, *L. rhamnosus* needs to be colonized in the intestine to a certain amount [3]. However, *L. rhamnosus* is sensitive to external factors, e.g., low pH of gastric acid and high content of bile salt [4,5], which leads to a relatively low survival rate in intestine environment, thereby limiting its wide application. In addition, the probiotics are easily damaged during storage and transportation process.

Microencapsulation of probiotics has been demonstrated to be an effective strategy for the protection of bacteria from their surrounding environmental conditions, thereby helping them colonize in the intestine [6]. Several studies have shown the benefits of microencapsulation on long-term storage stability and the protection of probiotics under gastrointestinal conditions [7–9]. Several methods have been applied for the encapsulation of probiotics, such as extrusion method [10], emulsification method [11], and gelation method [12]. Although internal emulsification/gelation technique has been commonly used for the encapsulation of bioactive compounds [13], the study using this method for the encapsulation of probiotic bacteria has been rarely reported. Proteins, starch and polysaccharides are often used as encapsulation wall materials [14–16]. These materials should have the characteristics of non-toxicity, no side effects, good biocompatibility, good film-forming property, and no reaction with core materials. Whey protein, a by-product of cheese production, is a popular encapsulating material,

mainly containing β-lactoglobulin serum albumin and immunoglobulin [17]. Due to the amphiphilic nature of whey protein [18], it can form good emulsions and anti-gastric hydrogels [19]. However, the wall material with a single constituent is difficult to provide adequate coating for the core materials, leading to a failure in the achievement of specific effects and functions, such as resistance to the external degradation and the long-term storage [20]. It was reported that use of the complex wall materials by combining whey protein with polysaccharides could avoid the disadvantages of using a single wall material [9]. Pectin is a good choice for this purpose, furthermore, it possesses a variety of biological activities [21].

In our previous study, a strain of *L. rhamnosus* ZFM231 with good exopolysaccharide (EPS)-producing capacity was isolated and identified [22]. The EPS produced by this strain possess good probiotic effect, such as hypolipidemic and antioxidant activities, gut microbiota-regulating and colitis-alleviating effects [7,22,23]. Therefore, it is important to develop a method to improve the viability of *L. rhamnosus* ZFM231 strain in the gastrointestinal tract and increase its stability, so as to provide better probiotic effect. To this end, an internal emulsification/gelation technique was employed for the encapsulation of *L. rhamnosus* ZFM231 strain. Herein, the fabrication of double layer *L. rhamnosus* ZFM231 microcapsules using whey protein as the inner wall material and pectin as the protective wall material is reported. The tolerance of *L. rhamnosus* ZFM231 microcapsules in the simulated gastric fluid (SGF), the release in the simulated intestinal fluid (SIF), storage and thermal stabilities were also investigated.

2. Materials and methods

2.1 Cultivation of *L. rhamnosus ZFM231*

L. rhamnosus ZFM231 was isolated from fresh milk, and has been deposited in the China Center for Type Culture Collection (CCTCC) under accession number NO. CCTCC M 2019883. *L. rhamnosus* ZFM231 was inoculated on MRS agar, and cultured at 37 °C for 48 h. A colony with good growth was selected and inoculated into MRS broth for 24 h at 37 °C. After 3 generations of activation, the bacteria were collected by centrifugation (4 °C, 8000 rpm, 10 min), the concentration of bacterial solution was adjusted to 10^6-10^8 CFU mL^{-1}, and inoculated into MRS broth with 2% (v/v) inoculation amount at 37 °C for 24 h.

2.2 Microencapsulation of *L. rhamnosus* ZFM231

Microencapsulation of the bacteria was carried out referring to a published method with slight modifications [24]. In brief, whey protein solution (4%-12%) was stirred (800 rpm) at 45 °C for 2 h, and heated to 80 °C and stirred for another 30 min, then allowed to stand at 4 °C overnight after cooling on an ice water bath. The bacteria were collected by the centrifugation (10000 rpm, 10 min) of above bacterial suspension, and the concentration of the bacteria was adjusted to about 10^9-10^{10} CFU mL^{-1} with normal saline, which was named as free bacteria solution. The free bacteria solution was mixed with whey protein solution in a ratio of 1:15 (v/v), which was named as bacteria-whey mixture. The mixture of water and soybean oil with a certain ratio (1:1-1:5, v/v) was named as water-oil mixture. The bacteria-whey mixture was mixed with water-oil

99 mixture at a ratio of 3.5:1, followed by the addition of $CaCl_2$ (final concentration 0.01 %,
100 w/v) and glucolactone (final concentration 0.4%, w/v). The resulting solution was
101 heated at 40 °C for a certain time (1-5 h) at a certain stirring speed (200-1000 rpm). The
102 precipitate was obtained by centrifugation at 8000 rpm for 6 min.

103 2.3 Single factor analysis

104 The effects of whey protein concentration (4%, 6%, 8%, 10%, 12%), stirring speed (200,
105 400, 600, 800, and 1000 rpm), emulsification time (1 h, 2 h, 3 h, 4 h and 5 h) and water-
106 oil ratio (1:1, 1:2, 1:3, 1:4, 1:5) on the encapsulation efficiency were studied using
107 single factor tests.

108 2.4 Response surface methodology (RSM)

109 On the basis of the results of single factor tests, the influences of the four factors,
110 including whey protein concentration (*A*), stirring speed (*B*), emulsification time (*C*)
111 and water-oil ratio (*D*) on the encapsulation efficiency were further investigated by
112 RSM. Design expert 10 software was used for the experimental design and analysis,
113 the levels of each factor were shown in Table 1. The design included 27 experimental
114 points, in which the central experiments were repeated three times.

115 2.5 Selection of protective agent of microcapsules

116 During freeze-drying, wet single-layer microcapsules might be damaged, resulting in a
117 reduced encapsulation efficiency. This reduction can be improved by adding a
118 protective agent to form another layer of coating on the surface of microcapsules, which

119 can also avoid the rapid hydrolysis of whey protein by gastric acid in the stomach,
120 thereby protecting the bacteria. In this study, trehalose, pectin and gelatin were screened
121 to select the optimal protective agent. The solution of wet single-layer microcapsules
122 prepared under the above optimal processing conditions was mixed with the solution
123 of protective agent (0.1 g mL^{-1}, trehalose, pectin or gelatin) with a ratio of 2:5 (v/v),
124 and stirred for 15 min. The wet double-layer microcapsules were obtained by
125 centrifugation (8000 rpm, 6 min). Dry double-layer microcapsules of *L. rhamnosus*
126 ZFM231 were obtained by freeze-drying. The protective effect of protective agents on
127 microcapsules was evaluated by measuring the encapsulation efficiency and
128 microcapsule particle size.

129 2.6 Determination of encapsulation efficiency

130 Encapsulation efficiency was measured according to Annan's method with
131 modification [25]. Briefly, 0.2 g of microcapsules were added to 2 mL of 0.5 M sodium
132 citrate solution, and underwent vortex shaking until the microcapsules were basically
133 dissolved. The resulting mixture was shaken at 37 °C for 1 h in a thermostatic shaker
134 (180 rpm). The homogenate (1 mL) was taken for gradient dilution, the yielding diluted
135 solution (100 μL) was spread on MRS agar and cultured at 37 °C for 48 h. The viable
136 bacteria were counted and the encapsulation efficiency was calculated using equation
137 (1):

138 $$\text{Encapsulation efficiency (\%)} = \frac{N}{N_0} \times 100 \qquad (1)$$

139 where N is the number of viable *L. rhamnosus* ZFM231 released from the

140 microcapsules, N_0 is the number of viable cells in the suspension before
141 microencapsulation.

142 2.7 Particle size and ζ-potential analysis of microcapsules

143 The particle size and ζ-potential of the microcapsules were determined using a laser
144 particle size analyzer Mastersizer 2000 (Malvern, Germany). The samples were diluted
145 using distilled water, loaded into a capillary cell and analyzed at 25 °C, ζ-potential was
146 measured at pH 6. The measurements were performed in quintuplicate.

147 2.8 Morphological observation of microcapsules

148 The morphology of the wet microcapsules was analyzed using an optical microscope
149 (Nikon ECLIPSE Ti-S, Japan). The morphology of the dried microcapsules was
150 evaluated using a Phenom ProX Desktop Scanning Electron Microscope (Phenom
151 Scientific, Netherlands).

152 2.9 FT-IR spectra and XRD pattern analysis

153 FT-IR spectra of the whey protein, pectin, probiotic-loaded microcapsules and the
154 control were recorded on a Nicolet iS50 infrared spectrometer (Thermo Fisher, USA)
155 using a KBr pellet method scanning in the range of 400-4000 cm^{-1} [26]. XRD pattern of
156 the probiotic-loaded microcapsules and the control were performed using an X-ray
157 diffractometer (Rigaku Ultima 4, Japan), following a published method [27], the test
158 conditions were as follows: Cu radiation of wavelength 1.5406 Å, pipeline pressure 40
159 kV, angle range 3-80 ° (2θ), scanning speed 0.5°/min.

160 2.10 Survival of the bacteria under the simulated gastrointestinal conditions

161 The survival of the bacteria in microcapsules exposed to the simulated gastrointestinal
162 environment was measured according to a previous report with minor modification [28].
163 SGF was prepared by adjusting the pH value of saline to 2.0 using 0.1 M HCl, followed
164 by the addition of pepsin (final concentration 1%, w/v). SIF was prepared by adjusting
165 pH of 0.05 M KH_2PO_4 to 7.4 using 0.1 M NaOH, followed by the addition of trypsin
166 to reach a final concentration of 1% (w/v). The SGF and SIF solutions were passed
167 through a 0.22 μm membrane before use.
168 Microcapsules (1 g) were evenly dispersed in SGF or SIF (50 mL) respectively, and
169 then placed in a constant temperature shaker (200 rpm, 37 °C) for digestion to simulate
170 the gastrointestinal condition. Samples (1 mL) were taken at 0, 30, 60, 90 and 120 min,
171 respectively, for bacterial counting [29].

172 2.11 Storage stability of microcapsules

173 The double-layer wet microcapsules and freeze-dried microcapsules prepared under the
174 optimized conditions were stored at 4 °C for 28 days and at 25°C for 14 days
175 respectively. The unencapsulated bacterial sample was used as a control.

176 2.12 Thermal stability of microcapsules

177 The survival of the bacteria in microcapsules exposed to the different temperature
178 conditions was measured according to a published report with minor modification [30].
179 Briefly, 0.5 g microcapsules or 0.5 mL *L. rhamnosus* ZFM231 bacteria suspension was

added into sterile tube respectively, evenly dispersed in 4.5 mL of phosphate-buffered saline. After incubation in water bath with different temperature (55, 65 and 75°C) for 10 min, the number of bacteria was counted.

2.13 Statistical analysis

One-way analysis of variance (ANOVA) was performed using SPSS version 25 (SPSS Inc., Chicago, IL, US) for comparison of the results. Duncan's multiple-range tests were used for analyzing the significance of the differences. If $p<0.05$, the differences were considered statistically significant. All the data were expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1 Optimization of encapsulation conditions

3.1.1 Single factor tests

As shown in Fig. 1A, the encapsulation efficiency of microcapsules increased first and then decreased with the increase of whey protein concentration. When the concentration of whey protein was 8%, the encapsulation efficiency of microcapsules reached the maximum, being 75.27%, which was significantly higher than those obtained at other concentrations ($p<0.05$). At relatively low whey protein concentration, the bacteria could not be encapsulated well. This may be due to the loose of the wall structure of the formed microcapsules, which allowed the bacteria to dissociate to the outside of the microcapsule. With the increase of the whey protein concentration, the formed microcapsule wall gradually became dense and tough, thereby improving the

encapsulation efficiency. The particle size of microcapsules increased significantly with increasing the concentration of whey protein (p<0.05), which is possibly due to the formation of the thicker wall of the microcapsules. Hence, 8% of whey protein was chosen in the following single factor tests due to the best encapsulation efficiency and appropriate particle size of microcapsules.

The stirring speed was found to significantly influence the encapsulation efficiency and particle size of microcapsules (p<0.05) (Fig. 1B). The highest encapsulation efficiency and smallest particle size was observed when the stirring speed reached 800 rpm, being 70.10%, and 106.97 μm, respectively. At a lower stirring speed, incomplete emulsification would occur, resulting in the continuous aggregation of small single body fluid droplets, thus forming large emulsion droplets which led to the large particle size and low encapsulation efficiency. Appropriate elevated stirring speed improved the emulsifying effect, and consequently resulting in the reduction of particle size and the improvement of encapsulation efficiency. However, too high stirring speed could cause the damage of the network structure of the composite wall and demulsification. Thus, 800 rpm was chosen as the optimal stirring speed.

As shown in Fig. 1C, encapsulation efficiency and particle size of microcapsules increased significantly with the increase of emulsification time up to 3 h (p<0.05), thereafter encapsulation efficiency decreased (p<0.05), while the particle size remained almost unchanged. It was likely that the emulsification gradually increased with increasing the emulsification time at the beginning, leading to an increase of encapsulation efficiency. However, when the emulsification time exceeded 3 h, there

might be adhesion in the microcapsules, which caused aggregation, leading to a decrease of encapsulation efficiency. Thus, emulsification time of 3 h was chosen as the optimal time.

The effects of water/oil ratio on encapsulation efficiency and particle size of microcapsules are presented in Fig. 1D. When the water/oil ratio was 1:4, the encapsulation efficiency was the highest, being 70.10%. The particle size of microcapsules decreased with the decrease of water/oil ratio. This may be because when the water/oil ratio was high, the water component content in the formed microcapsules was high, resulting in the thinness of microcapsule wall, which led to a reduction in the protection on the microcapsules.

3.1.2 RSM analysis

The encapsulation conditions of *L. rhamnosus* ZFM231 were further optimized by RSM based on the results of single factor tests. Regression analysis on the response surface experimental results, presented in Table 1, was performed using Design Expert 10 software, providing the following quadratic multiple regression equation:

$$\text{Encapsulation efficiency } (\%) = 85.16 - 1.92A + 1.71B - 0.34C + 1.02D + 2.07AB + 5.79AC + 0.27AD + 1.9BC + 2.32BD - 3.72CD - 6.4A^2 - 11.19B^2 - 7.15C^2 - 9.39D^2 \quad (2)$$

The results and ANOVA of the model are presented in Table 2. The *F*-value of the model was 87.62 with an extremely low *p*-value (<0.0001), suggesting that the model was adequate. The *p*-value for lack of fit (0.0913) indicated the insignificance of lack of fit compared with the pure error. The value of the regression coefficient R^2 was 0.9542,

245 implying that the model fits the test process well, while R^2_{adj} (0.9007) explained more
246 than 90 % of the reliability. The good fitting effect of the model indicated that the high
247 reliability and accuracy of the test.
248 The optimal level of the four variables and their interactions were visualized by the
249 three-dimensional RSM and the count plots in function of two variables (Fig. 2). The
250 elliptical shape of the contour plots indicated that the interactions between the variables
251 were significant. The optimal values of the variables calculated by the equation model
252 were: whey protein concentration 7.62%, stirring speed 811.52 rpm, the emulsification
253 time 2.89 h and the water/oil ratio 1:4.08, the encapsulation efficiency of microcapsule
254 reached 85.45%. Considering the practicality of operation, the optimized encapsulation
255 conditions were ascertained as: whey protein concentration 7.6%, stirring speed 800
256 rpm, emulsification time 2.9 h and water/oil ratio of 1:4. Three parallel experiments
257 were carried out under the optimized condition, and the encapsulation efficiency was
258 determined to be 85.20±0.61%, which was very close to the predicted value by the
259 model, indicating the validation of the model. The particle size of microcapsules
260 prepared under the optimum condition was found to be 123.7±2.6 μm.

261 3.2 Selection of protective agent of microcapsules

262 Using protective agent to form a protective layer on the outer wall of microcapsules is
263 an effective mean to reduce the damage of microcapsules during freeze-drying. As
264 shown in Fig. 3A, the effect of pectin as a protective agent was superior to those of
265 trehalose and gelatin (p<0.05). Pectin could form a gel on the outer surface to protect

266 whey protein on the surface of the microcapsules, which ensured the integrity of the
267 microcapsules. In addition, pectin could also avoid the degradation of microcapsules
268 by gastric acid in the stomach. Effects of microcapsule/pectin ratio on the encapsulation
269 efficiency and the particle size of the microcapsules are illustrated in Fig. 3B. At a
270 microcapsule solution/pectin solution ratio of 2:4, a maximum encapsulation efficiency
271 of microcapsules was achieved (89.46±0.82%), with a particle size of 172±1.8 μm. The
272 particle size distribution of microcapsules was shown in Fig. 3C. The microcapsules
273 were found to possess normal particle size distribution, with diameter of mainly 171-
274 174 μm, indicating that the microcapsules prepared by this method had good size
275 uniformity distribution.
276 Encapsulation efficiency is an important index for the evaluation of the success of
277 microcapsule preparation. Similar encapsulation efficiency had been reported by some
278 researches. For instance, microencapsulated *L. rhamnosus* was prepared with an
279 encapsulation efficiency of 88.88% [20]; a dry encapsulated *L. casei* was obtained with
280 an encapsulation efficiency of 83.25-84.34% [5]. There were also some documented
281 reports, in which encapsulation efficiency was relatively low. Chávarri et al.
282 microencapsulated a probiotic and prebiotic by alginate-chitosan with the encapsulation
283 efficiency of 40.2% [31]; Annan et al. encapsulated *Bifidobacterium adolescentis* using
284 alginate-coated gelatin and obtained an encapsulation efficiency of 41.1% [24]. The
285 particle size of the microcapsules might affect the texture and sensory quality of the
286 food [32], and the particle size of microcapsules was quite different in different studies.
287 Neuenfeldt et al. reported that the particle size of *L. rhamnosus* microcapsules ranged

288 from 1.74 to 6.29 μm [33], while Azam et al. found the particle sizes of *L. rhamnosus*
289 microcapsules ranged from 400 μm up to 3 mm [14]. It was reported that the particle
290 size of *L. plantarum* microcapsules was 196.2 nm when whey protein and Gum Arabic
291 were used as wall materials [9]. In general, the double-layer microcapsules have a
292 superior protection to the core material compared with single-layer structure. Zhang et
293 al. used the mixture of sodium alginate/whey protein as the first layer and cellulose
294 nanocrystal coating as an outer layer, the results showed that the single-layer
295 microcapsules were not as effective as double-layer microcapsules in protecting the
296 bacteria during the long-term storage and upon exposure to the simulated
297 gastrointestinal fluid [5].

298 3.3 Characterization of *L. rhamnosus* ZFM231 microcapsules

299 3.3.1 ζ-potentials

300 ζ-potential can be used to predict the interfacial reactions between the microcapsules
301 and the surroundings, and can reflect the stability of the microcapsules in food systems
302 [34]. The ζ-potential of the double-layer microcapsules and the mono-layer
303 microcapsules were -18.36 mV and -4.38 mV respectively. The negative ζ-potential
304 might be associated with the dissociation of protons from all carboxyl groups [35]. The
305 ζ-potential of double-layer microcapsules was significantly lower than that of the
306 mono-layer microcapsules (p<0.05), which may be due to the $–COO^-$ groups of pectin
307 on the outer protective layer, resulting in greater repulsion between the components
308 [36]. This result agrees with that of Nasiri et al. who reported that the microcapsules
309 using galactomannan mucilage extracted from wild sage seed as the second layer wall

material possessed a ζ-potential of -17.69 mV, being much lower than that of the mono-layer microcapsules with sodium alginate as wall material (-2.70 mV) [34]. Sharifi et al. used whey protein isolate and gum Arabic for the co-encapsulation of *L. plantarum*, and revealed that the charge density of gum Arabic was higher than whey protein isolates and the surface of coacervates was negatively charged [9].

3.3.2 FT-IR analysis

FT-IR spectra of the whey protein, pectin and microcapsules are presented in Fig. 4A. Whey protein is amphiphilic substance, the characteristic peaks at 3400 cm^{-1} revealed the hydrophilic hydroxyl groups, and the peak at 2960 cm^{-1} was ascribed to the saturated C-H stretching vibration, the band at 1640 $cm^{-1}$was assigned to stretching vibration of carbonyl group (Amide I) [37]; the peak at 1550 cm^{-1} could be attributed to the bending vibration of N–H (Amide type II); while the peak at 1230 cm^{-1} was assigned to C–N bond (Amide type III) [38]. The spectrum of pectin revealed typical characteristic peaks for carbohydrate. The peak at 1740 cm^{-1} was caused by stretching vibration of esterified carbonyl (C=O) and 1640 cm^{-1} was caused by stretching vibration of carboxylate anion (COO^-), and the multiple weak vibrations observed between 950 cm^{-1} and 1300 cm^{-1} also revealed the presence of carboxylate anion [39]. Due to the electrostatic interaction between whey protein and pectin, the absorption of the carbonylamide region in the IR spectra of the probiotic-loaded microcapsules and whey protein-pectin complex (control) changed when compared to free whey protein and pectin, such as the COO^- tensile vibrations at 1530 cm^{-1}, as well as the slight changes in the signals of the N-H at 1550 and 1640 cm^{-1}. The -OH peaks of whey protein, pectin, probiotic-loaded

microcapsules and the control were significantly shifted, indicating the existence of hydrogen bonding interactions among them. The characteristic peak produced by the asymmetric stretching vibration of saturated C-H had more significant enhancement in the control, indicating that there was a hydrophobic interaction between whey protein and pectin, which disappeared after the bacteria was loaded, possibly due to the functional groups in the cell wall components. Overall, the formation of the probiotic-loaded microcapsules was associated with the electrostatic interactions, hydrophobic-hydrophilic interactions, and hydrogen bonding interactions among whey protein, pectin and the bacteria.

XRD analysis shows that the patterns of probiotic-loaded microcapsules and the control were similar, exhibiting a wide dispersion peak at 20-40° (Fig. 4B and 4C), which indicated the presence of polycrystalline and amorphous coexisting systems in both systems.

3.3.3 Morphological characterization

As can be seen in Fig. 5A, the microcapsules were small but visible to the naked eyes. The magnified image showed that the microcapsules appeared milky white and spherical. In the optical microscope image (Fig. 5B), the microcapsule was of a similar spherical shape, and an obvious two-layer shell structure with multinuclear was observed. This finding is similar to those in some studies [40–42]. It was reported that *Bifidobacterium* microcapsules, prepared by complex coacervation using gelatin and gum Arabic as wall materials, presented round shapes with uniform distribution of the microorganisms in the interior [41]. The SEM analysis indicated that the shape of

354 microcapsules was irregular, and the surface presented porous and rough appearance
355 (Fig. 5C), which may be related to the reduction of water during freeze-drying. This
356 result is similar to those of some reports [43,44]. The double layer structure with thick
357 wall and a complete spherical structure of the first layer encapsulation of the incomplete
358 microcapsule are clearly observed in Fig. 5D. Adding protective agent as the second
359 wall material increased the thickness of the outer wall of the microcapsules, which was
360 similar to the result of Rajam and Anandharamakrishnan, who encapsulated *L.*
361 *plantarum* using Fructooligosaccharides and whey protein [45].

362 3.4 Survival under the simulated gastrointestinal conditions

363 As illustrated in Fig. 6A, the number of viable bacteria of both microencapsulated and
364 free *L. rhamnosus* ZFM231 decreased in SGF. After being treated in SGF for 90 min,
365 the viability of free bacteria decreased from 9.59 to 5.60 log (CFU g^{-1}), indicating *L.*
366 *rhamnosus* ZFM231 was sensitive to the acidic environment. As reported in many
367 articles, the free probiotics are easily destroyed by gastric acid. Sohail et al. [46]
368 reported that *L. acidophilus* lost more than 6 log CFU g^{-1} in SGF at pH 2.0 after 20 min.
369 Under the same conditions, the decreasing rates of the viability for the single-layer and
370 double-layer microencapsulated bacteria were significantly lower than that of free *L.*
371 *rhamnosus* ZFM231 (p<0.05), decreasing from 9.51 to 6.32 log (CFU g^{-1}) and from
372 9.48 to 7.52 log (CFU g^{-1}), respectively. Similarly, Santos et al. found that *L.*
373 *acidophilus* microcapsules encapsulated with inulin showed less log reduction when
374 compared to the unencapsulated probiotics, only 2.0 log CFU g^{-1} reduction for the

375 encapsulated probiotics with spray drying was observed [47]. Obviously,
376 microencapsulation is an effective way to protect probiotics from the damage under
377 acidic conditions, which allows more bacteria to reach the intestine to play the probiotic
378 effect.

379 A rapid release of *L. rhamnosus* ZFM231 from the double-layer microcapsules in SIF
380 was observed at the first 30 min, thereafter the release slowed down significantly (Fig.
381 6A). By 60 min, the number of live bacteria reached about 9.06 log (CFU g^{-1}). After
382 treatment in SIF for 90 min, the number of viable bacteria was 9.10 log (CFU g^{-1}), and
383 the release rate was 86.56%. Afterwards, the number of released live bacteria remained
384 almost unchanged, indicating that the microencapsulation in this study had good enteric
385 solubility. Similar findings have been reported, encapsulated *L. rhamnosus* with
386 Maillard reaction products of whey proteins and isomaltooligosaccharide could be
387 released from microspheres to reach a viable bacterial number of 9.13 log CFU g^{-1} in
388 60 min [20]. The number of *L. rhamnosus* GG released from whey protein microcapsule
389 reached 6.2 log CFU g^{-1} after 5 min, the complete release was achieved to reach a viable
390 bacterial number of around 9 log CFU g^{-1} following 30 min incubation in the simulated
391 intestinal fluid [48].

392 *3.5 Storage stability of microcapsules*

393 Storage stabilities of the wet microencapsulated and freeze-dried microencapsulated *L.*
394 *rhamnosus* ZFM231 prepared under the above optimal condition were evaluated by
395 viable bacteria counting during the storage at 4 °C for 28 days and 25 °C for 14 days,

396 respectively, in comparison with free *L. rhamnosus* ZFM231. As shown in Fig. 6B and
397 C, the number of viable bacteria in all groups decreased with the increase of storage
398 time, and the decreasing rate of viable bacteria number in the two encapsulated groups
399 was significantly lower than that of free *L. rhamnosus* ZFM231 group. This result is
400 similar to that reported by Mozaffarzogh et al. [49]. After stored at 4 °C for 28 days,
401 the viable bacteria number of the unencapsulated group decreased from 10.59 to 6.20
402 log (CFU mL^{-1}) with a loss of 4.39 log units. In contrast, viable bacteria number of the
403 wet encapsulated group decreased from 10.52 to 7.66 log (CFU g^{-1}) with a loss of 2.86
404 log units, and lyophilized powder group decreased from 10.48 to 9.02 log (CFU g^{-1})
405 with only a loss of 1.46 log units. After stored at 25 °C for 14 d, the viable bacterial
406 number of the bacteria group decreased from 10.52 to 7.21 log (CFU mL^{-1}) with a loss
407 of 3.31 log units, viable bacteria number of the wet encapsulated group decreased from
408 10.51 to 8.91 log (CFU g^{-1}) with a loss of 1.60 log units, and lyophilized powder group
409 decreased from 10.49 to 9.38 log (CFU g^{-1}) with a loss of 1.11 log units. These results
410 indicated the good protection of microcapsules for bacteria, and the dry microcapsules
411 provided better protection than the wet microcapsules, which could be because the
412 decrease in water content reduced the activity of bacteria or allowed them to remain
413 dormant [50]. This result was similar to those of encapsulated *Bifidobacterium lactis*
414 (Bb-12) prepared by complex coacervation with gelatin and gum Arabic [41].

415 *3.6 Thermal stability*

416 The outer wall of microcapsules can prolong the transmission of temperature and has a

protective effect on the probiotics inside. As shown in Fig. 6D, after treatment at 55 °C for 10 min, the viable bacterial number lost about 3.02 log units in the free bacteria group, when the temperature raised to 65 and 75 °C, almost all bacteria died. This finding is similar to that of Hu et al. [30]. With the protection of microcapsules, after treatment at 55 °C for 10 min, there was only a loss of 0.63 log units. As the temperature increased to 65 and 75 °C, the loss of viable bacteria was 2.03 and 2.89 log units, respectively. These results indicated that whey protein-pectin out-wall did play an important role in improving the structure and property of microcapsules.

4. Conclusion

In this study, double layer *L. rhamnosus* ZFM231 microcapsules were prepared using whey protein as wall material and pectin as a protective agent. Under optimized conditions, a good encapsulation efficiency of *L. rhamnosus* ZFM231 in dry microcapsules was achieved, being 89.67±0.82%, and the particle size of the obtained *L. rhamnosus* ZFM231 microcapsules was 172±1.80 μm. After exposure to SGF for 90 min, the bacterial count of microcapsule was 7.52 log (CFU g^{-1}) which was 1.9 orders of magnitude higher than that of free bacteria. The bacteria could be rapidly released from microcapsules in SIF and reached a release rate of 86.56% in 90 min. After storage at 4 °C for 28 d and 25 °C for 14 d, the bacteria count of the dry microcapsules was still 9.02 and 8.70 log (CFU g^{-1}), which was 2.8 and 1.49 orders of magnitude higher than that of free bacteria. The microcapsules could significantly improve the survival rate of *L. rhamnosus* ZFM231 in simulated gastrointestinal conditions and increase the storage

stability. Therefore, *L. rhamnosus* ZFM231 microcapsules investigated in this work has a potential application in functional foods and dairy products. However, investigation on the *in vivo* probiotic effects of *L. rhamnosus* ZFM231 microcapsules are required, which will be undertaken in the due course.

Conflict of interests

None declared.

Acknowledgements

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626 **Tables**

627

628 **Table 1**. Results of response surface experiments

No.	Whey protein (*A*)	Stirring speed (*B*)	Emulsification time (*C*)	Water/oil ratio (*D*)	EE (%)
1	-1	0	0	-1	71.68±1.30
2	0	-1	1	0	63.54±0.93
3	0	0	1	1	64.50±0.76
4	0	1	0	1	71.96±0.89
5	1	0	0	1	66.35±1.21
6	0	0	0	0	84.50±1.11
7	0	0	-1	-1	63.86±1.39
8	0	0	0	0	85.00±1.34
9	0	-1	0	-1	64.50±0.98
10	0	-1	0	1	61.95±0.90
11	-1	0	-1	0	79.96±2.01
12	0	0	-1	1	74.46±1.20
13	-1	-1	0	0	67.38±0.98
14	0	1	0	-1	65.23±0.90
15	0	0	0	0	85.97±1.45
16	1	0	1	0	77.51±1.20
17	0	1	-1	0	65.05±1.24
18	-1	0	0	1	72.00±1.69
19	0	-1	-1	0	68.39±1.29
20	1	0	0	-1	64.96±1.43
21	1	-1	0	0	61.90±1.14
22	-1	0	1	0	69.90±1.50
23	1	0	-1	0	64.40±1.59
24	0	1	1	0	67.82±1.23
25	-1	1	0	0	67.64±0.88
26	0	0	1	-1	68.76±0.80
27	1	1	0	0	70.43±1.00
Level (-1)	6	600	2	1:3	
Level (0)	8	800	3	1:4	
Level (1)	10	1000	4	1:5	
Unit	%	rpm	h	v:v	

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632 **Table 2**. Analysis of variance results of regression model

Source	Sum of squares	DF	Mean square	*F*-value	*p*-value	Significant
Model	1226.71	14	87.62	17.84	<0.0001	**
A	44.12	1	44.12	8.99	0.0111	*
B	34.92	1	34.92	7.11	0.0205	*
C	1.39	1	1.39	0.28	0.6039	
D	12.46	1	12.46	2.54	0.1371	
AB	17.1	1	17.1	3.48	0.0867	
AC	134.21	1	134.21	27.33	0.0002	**
AD	0.29	1	0.27	0.06	0.8133	
BC	14.52	1	14.52	2.96	0.1112	
BD	21.53	1	21.53	4.38	0.0582	
CD	55.2	1	55.2	11.24	0.0057	*
A^2	218.68	1	218.68	44.53	<0.0001	**
B^2	668.22	1	668.22	136.08	<0.0001	**
C^2	272.53	1	272.53	55.5	<0.0001	**
D^2	470.33	1	470.33	95.78	<0.0001	**
Residual	58.93	12	4.91			
Lack of Fit	57.81	10	5.78	10.35	0.0913	
Pure Error	1.12	2	0.56			
Cor Total	1285.64	26				

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634

635 **Figure captions**

636 **Fig. 1.** Effect of whey protein concentration (A), stirring speed (B), emulsification time
637 (C) and water/oil ratio (D) on the encapsulation efficiency in the single factor tests.
638 There were significant difference between the groups labelled with different lowercase
639 letters.

640 **Fig. 2.** Response surface plots and contour of the effects on encapsulation efficiency.
641 (A) Whey protein concentration and stirring speed; (B) Whey protein concentration and
642 emulsification time; (C) Whey protein concentration and water/oil ratio; (D) Stirring
643 speed and emulsification time; (E) Stirring speed and water-oil ratio; (F) Emulsification
644 time and water-oil ratio.

645 **Fig. 3.** The effects of protective agent (A), and the microcapsule/pection ratio on the
646 encapsulation efficiency (B); (C) Particle size distribution of the microcapsules. There
647 were significant differences between the groups labelled with different lower case
648 lteeres.

649 **Fig. 4.** Fourier transform infrared spectroscopy of microcapsules (A); X-ray
650 diffractometer of microencapsulated *L. rhamnosus* ZFM231(B) and the bacterial-
651 unloaded microcapsue control (C).

652 **Fig. 5.** Morphological observation of microcapsules. Image by digital camera (A);
653 Image by optical microscope (B); Image of complete microcapsules by SEM (C); Image
654 of incomplete microcapsules by SEM (D).

655 **Fig. 6.** Survival of encapsulated *L. rhamnosus* ZFM231 in SGF and release in GIF (A);
656 Viability of microencapsulated *L. rhamnosus* ZFM231 during storage at 4 ˚C for 28

657 days (B) at 25 ℃ for 14 days (C); Thermal ability of microencapsulated *L. rhamnosus*

658 ZFM231 under different temperature (D).

659

Fig. 1

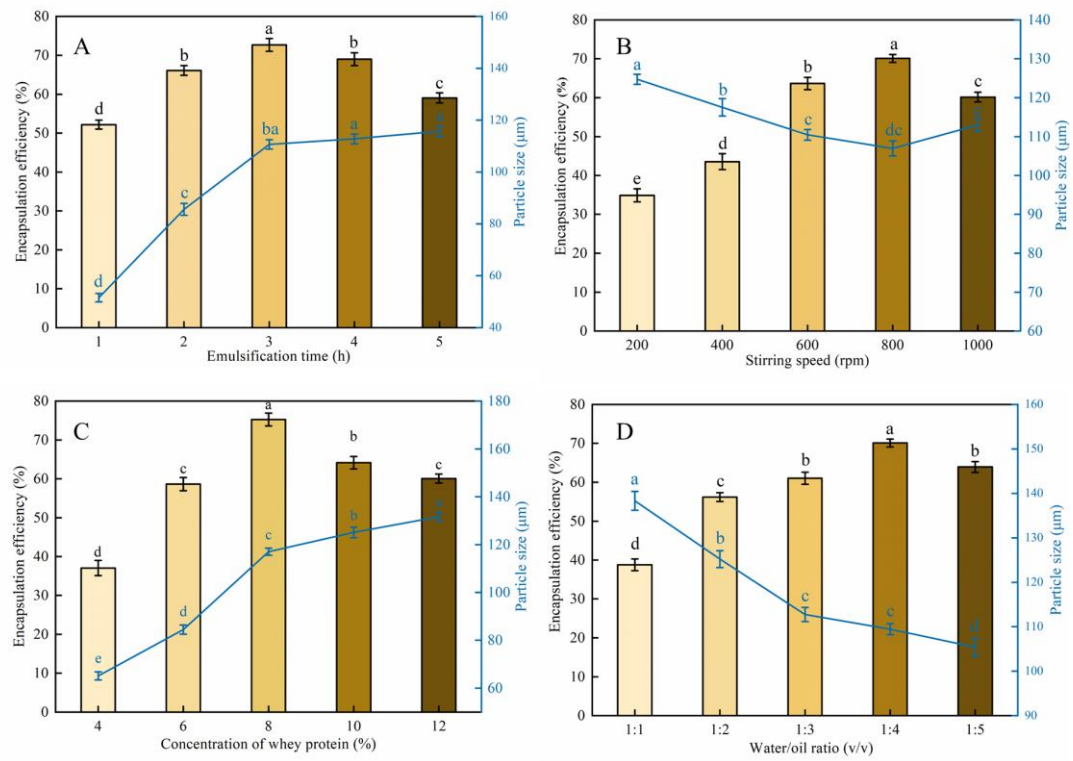
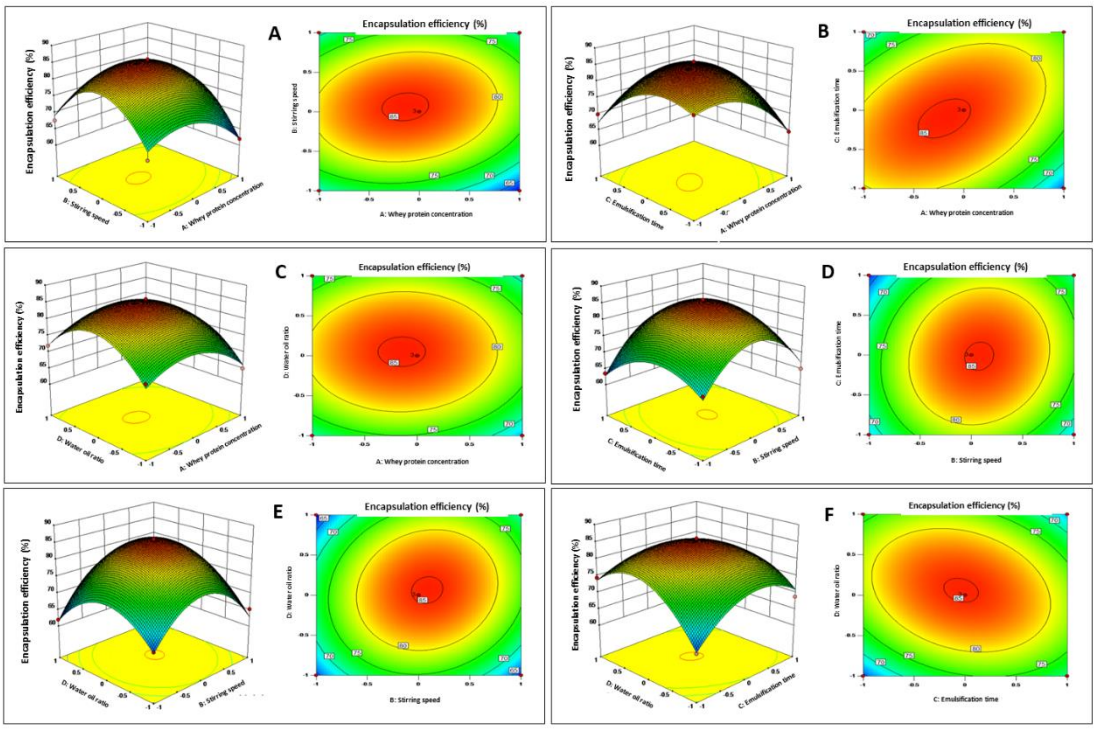
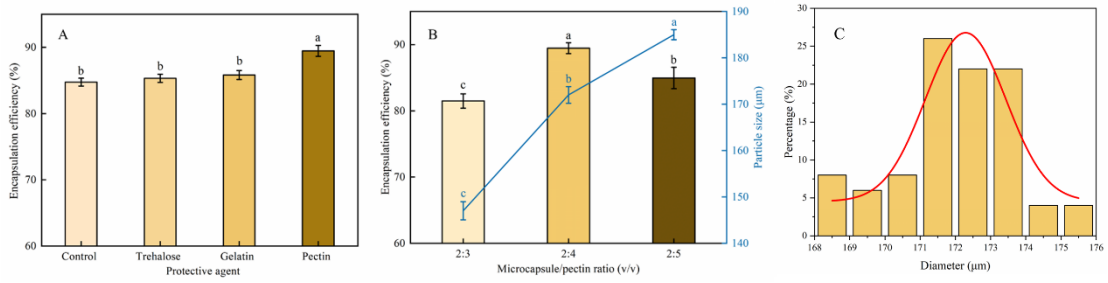

A
Encapsulation efficiency (%)
Particle size (μm)
Emulsification time (h)
B
Stirring speed (rpm)
C
Concentration of whey protein (%)
D
Water/oil ratio (v/v)

Fig. 2


A
Encapsulation efficiency (%)
Encapsulation efficiency (%)
B: Stirring speed
A: Whey protein concentration
B
Encapsulation efficiency (%)
Encapsulation efficiency (%)
C: Emulsification time
A: Whey protein concentration
C
Encapsulation efficiency (%)
Encapsulation efficiency (%)
D: Water oil ratio
A: Whey protein concentration
D
Encapsulation efficiency (%)
Encapsulation efficiency (%)
C: Emulsification time
B: Stirring speed
E
Encapsulation efficiency (%)
Encapsulation efficiency (%)
D: Water oil ratio
B: Stirring speed
F
Encapsulation efficiency (%)
Encapsulation efficiency (%)
D: Water oil ratio
C: Emulsification time

668 **Fig. 3**



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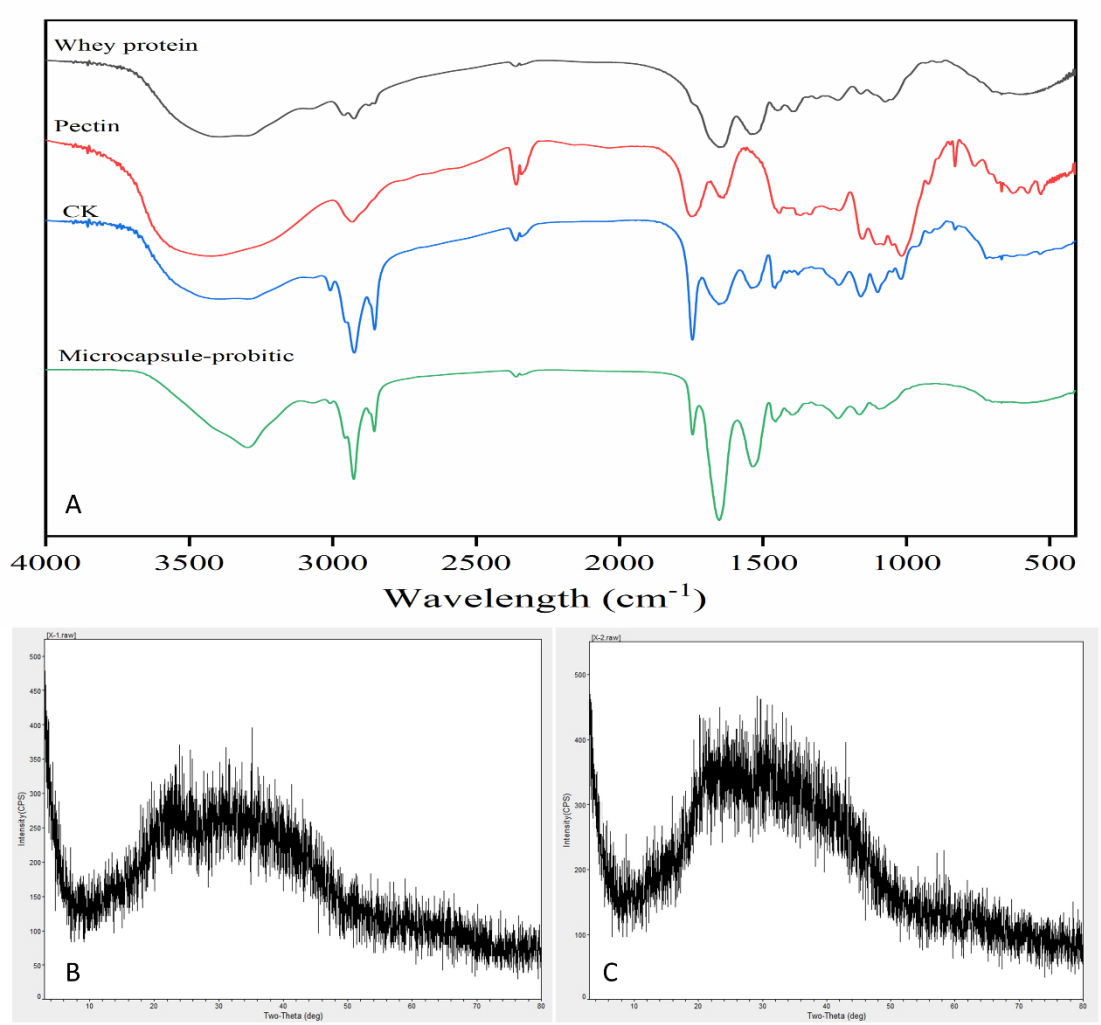
671

672 **Fig. 4**



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676 **Fig. 5**

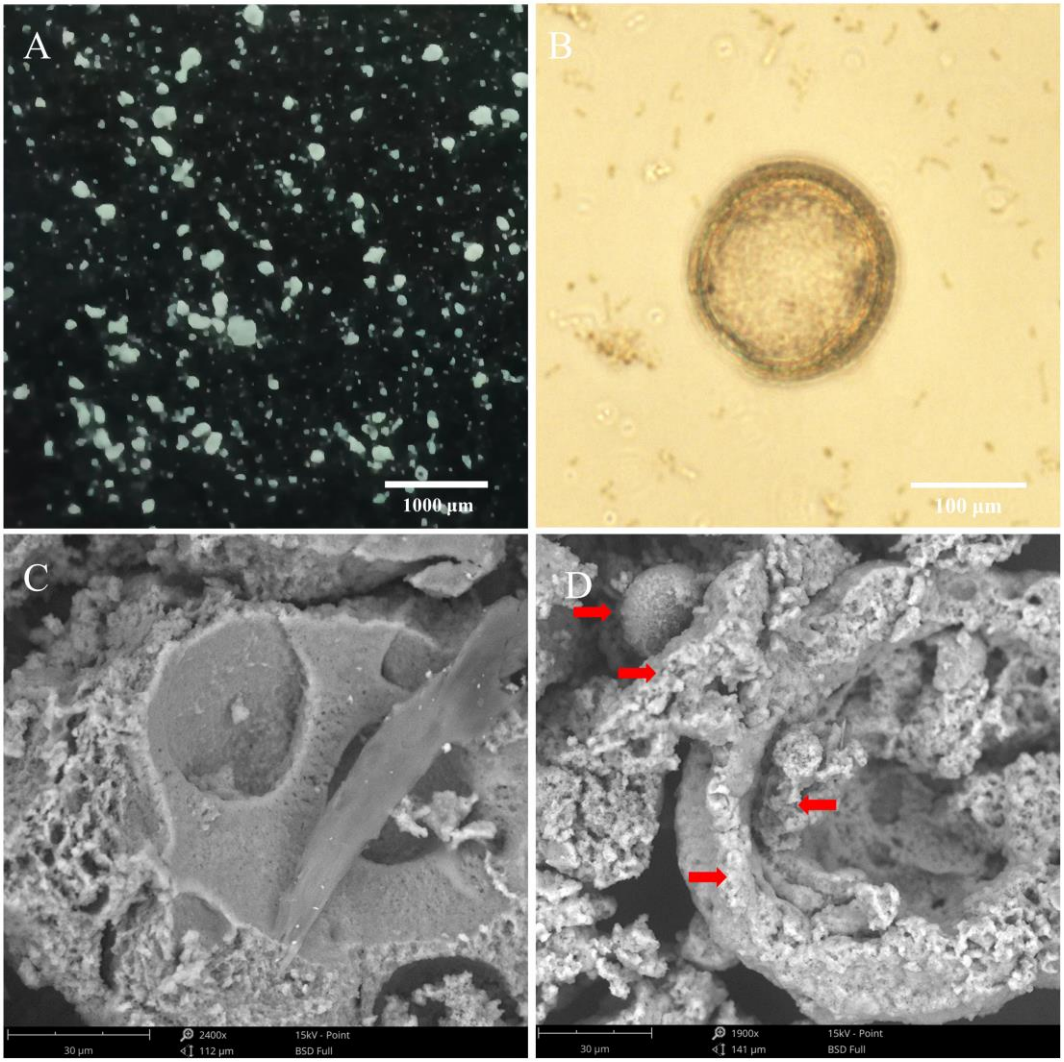

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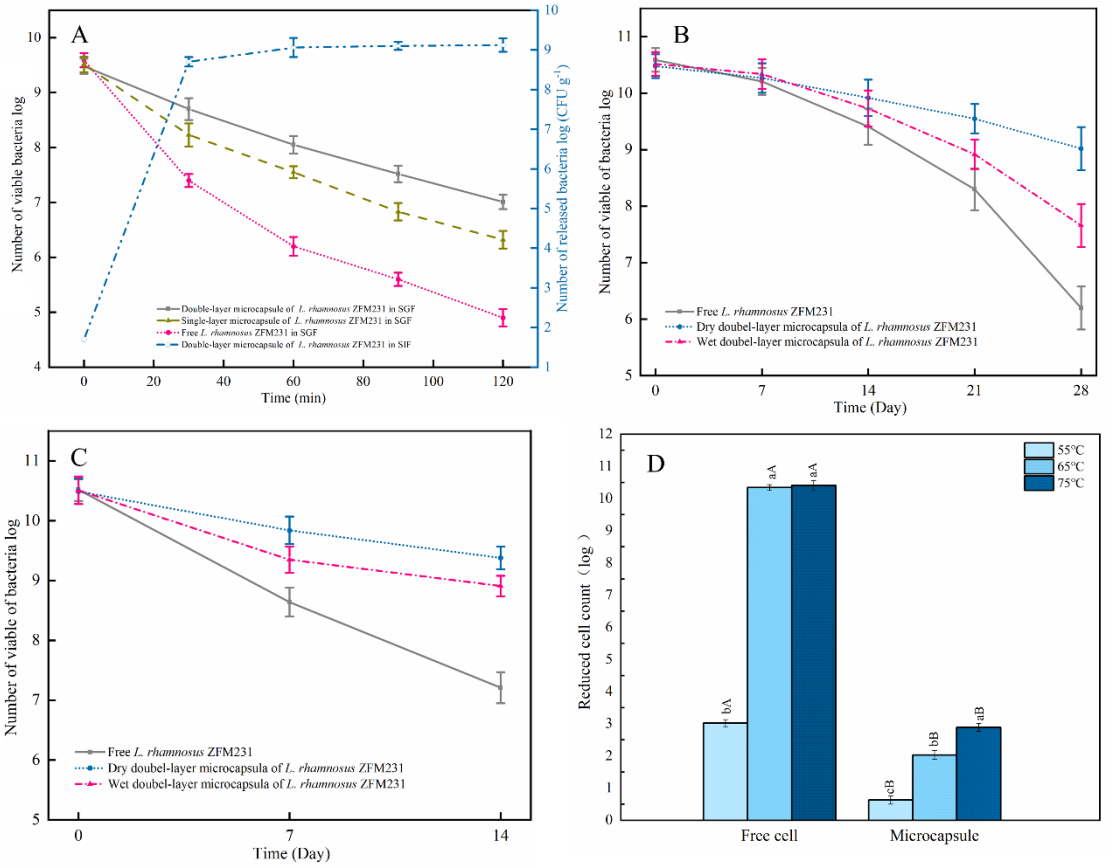
Fig. 6


A
Number of viable bacteria log
Number of released bacteria log (CFU g-1)
Time (min)
Double-layer microcapsule of L. rhamnosus ZFM231 in SGF
Single-layer microcapsule of L. rhamnosus ZFM231 in SGF
Free L. rhamnosus ZFM231 in SGF
Double-layer microcapsule of L. rhamnosus ZFM231 in SIF
B
Number of viable of bacteria log
Time (Day)
Free L. rhamnosus ZFM231
Dry doubel-layer microcapsula of L. rhamnosus ZFM231
Wet doubel-layer microcapsula of L. rhamnosus ZFM231
C
Number of viable of bacteria log
Time (Day)
Free L. rhamnosus ZFM231
Dry doubel-layer microcapsula of L. rhamnosus ZFM231
Wet doubel-layer microcapsula of L. rhamnosus ZFM231
D
Reduced cell count（log）
55℃
65℃
75℃
bA
aA
aA
cB
bB
aB
Free cell
Microcapsule